Title: **Targeting the Dark Horse of complement: the first generation of functionally selective C5aR2 ligands**

Strapline: **Functionally selective C5aR2 ligands discovery**

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MAIN TEXT

Complement is not only critical to the removal of invading pathogens but, if dys-regulated, also contributes to a broad range of inflammatory diseases. However, mentioning of complement generally does not trigger too much excitement, as the prevailing opinion among immunologists and clinicians is that complement is fully explored. Recent work, however, demonstrated conclusively that this system is a key 'instructor' of adaptive immunity, that complement activation occurs unexpectedly within cells were it contributes to metabolic reprogramming required for immune cell effector function and that dys-regulation of such intracellular complement contributes to autoimmune disease.[1,2] Furthermore, over- or under-active complement not only causes chronic infection or autoimmunity but also contributes to neuronal diseases, developmental and behavioral disorders, cancer, and even to aging and learning.[1] These exciting new developments in the field underpin that complement's functions are more far-reaching than previously thought may that complement is an underexplored therapeutic target. Thus, what is now urgently needed are reagents that allow the field to dissect these novel complement activities in detail. In this issue of *Immunology and Cell Biology*, Croker *et al*., identified such reagents with the discovery of the first two functionally selective ligands for the anaphylatoxin C5a receptor C5aR2.[3]

Complement activation in serum via pathogen (or self-derived danger) sensing induces the proteolytic activation of C3 into C3a and C3b, and of C5 into C5a and C5b. C3b is an opsonin and mediates the phagocytic uptake of pathogens by scavenger cells such as neutrophils and macrophages and C5b leads to the generation of the so-called membrane attack complex and direct lysis of microbes. C3a and C5a are anaphylatoxins and required for the general inflammatory reaction via inducing migration and activation of immune cells (which all express anaphylatoxin receptors) to and at the side of infection.[1] C5a is considered the body's most potent chemoattractant and can bind to two distinct receptors, C5aR1 and C5aR2.[4,5] While the role of the C5a receptor 1 (C5aR1 or CD88) is currently better defined, both in human biology and in mouse models, and is commonly seen as a receptor that is pro-inflammatory and required for normal effector function of immune cells, the role of the C5a receptor 2 (C5aR2, or GPR77, or C5L2) in immunity is much more

controversial and less well understood. Both receptors are seven-transmembrane domain (7TM), G protein-coupled (GPC), receptors. C5aR1 is a classic GPCR and its stimulation leads to predominantly G□$_i$ activation with subsequent mitogen-activated protein kinase (MAPK) and specifically extracellular signal-regulated kinase 1 and 2 (ERK1/2) activation and signal induction.[6] C5aR2 is not coupled to G proteins but can interact directly with β-arrestins (Figure 1).[7] Functionally, the C5aR2 was initially defined as an anti-inflammatory receptor that could counter-act the pro-inflammatory actions of the C5aR1 by 'fishing away' or competing for C5a and, thus, was called a 'C5a decoy receptor'.[3] Extended subsequent work on C5aR2, however, demonstrated quickly that C5aR2 functions in its own right and can have pro- and anti-inflammatory activity that can be dependent or independent of concurrent C5aR1 stimulation.[4] C5aR2 signals, fully independent of C5aR1 signals, particularly strongly contribute to the cytokine storm observed in sepsis.[3] Thus, what is currently understood is that the C5aR2 is important in regulating immune and non-immune pathways and that its specific function is dictated by the cell on which it is expressed and on the context (development, infection, autoimmunity etc.) in which activation occurs. However, progress in better understanding the mechanisms underlying C5aR2 activity has been hampered to date by the lack of specific agonists and antagonists. With the discovery of first two functionally selective agonists/ligands for C5aR2 (P32 and P59) by Croker *et al*., this hurdle can now be overcome.

P32 and P59 were identified by the authors through screening a computationally designed library of 61 hexa-, hepta-, and octapetides based on the *C*-terminal sequence of human C5a using a ^{125}I-C5a displacement assay on C5aR1 or C5aR2 expressing membranes.[8] Interestingly, although P32 and P59 can bind to both C5aR1 and C5aR2, the peptides have neither blocking nor stimulating activity towards C5aR1 – with the reasons for this observation being currently unresolved. Both agonists, however, have clear and defined impact on C5aR2 activity: these ligands trigger recruitment of β-arrestin via C5aR2, partially inhibit C5a-mediated ERK1/2 activation, and specifically reduce lipopolysaccharide-stimulated interleukin (IL) 6 production in macrophages (Figure 1). Importantly, neither peptide induced C5aR1 mediated ERK1/2 activation, demonstrating specificity for C5aR2. Excitingly, P32 is also functional *in vivo* with no obvious 'off-target effects' as it inhibits C5a-driven neutrophil migration and activation in wild type but not in *C5ar2*$^{-/-}$ animals.

Critically, this work not only delivers much-desired new tools to define C5aR2 functions but simultaneously uncovers a novel role for C5aR2 in directly modulating the inflammatory response as it demonstrates that activation of this receptor selectively modulates IL-6 release from human macrophages in the absence of serum-derived C5a. Although connections between C5aR2 signaling have been made previously, it is somewhat surprising that these observations have not been followed up in more depths given the central role of IL-6 in a wide range of pathologies and our still limited knowledge about the regulation of this key cytokine.[9] Based on the findings in this current study, C5aR2 agonists could potentially become novel anti-inflammatory drugs to selectively modulate IL-6 without impairing other aspects of the immune system. Furthermore, Croker *et al*. observed that selective C5aR2 activation inhibits the C5a-mediated signaling capacity of macrophages, and C5a-mediated neutrophil mobilization, but did not completely block it. As such, C5aR2 agonists could have the unique ability to town down the hyper-inflammatory activity of C5a, without fully inactivating it – an approach that is now favored by the pharmaceutical industries because of the growing understanding that complement is also important to tissue regeneration during the post-inflammation repair phases.[1] Although these are clearly strong new scientific tools developed by Croker *et al*., it remains to be assessed whether P32 and/or P59 recapitulate all C5aR2-driven events when compared to C5a binding to this receptor.

Further, with the emergence of intracellular C5aR1 and/or C5aR2 receptor activity and the cell-specific effects of C5aR2 stimulation, an important challenge for future therapeutic application will be to target these agonists in a controlled spatial and possibly also temporal fashion to the diverse 'sites of C5aR2 action'.

CONFLICT OF INTEREST

CK has a pending USA/European patent application in regards to C5aR2 targeting.

FIGURE LEGENDS

Figure 1. Targeting C5aR2 in immune cell regulation. In macrophages (left half), P32 and P59 induce β-arrestin recruitment via C5aR2 (1) but inhibit C5aR1 and C5aR2 heterodimerization (2) driven by high C5a (the functional consequences of this latter effect are not known). The P32/P59 and C5aR2 interaction also reduces C5aR1-driven ERK1/2 activation (3) and LPS-TLR4-

mediated IL-6 production (4). As TLR4 also induces ERK1/2 activation, which is required for IL-6 production, C5aR2 may negatively control LPS-driven IL-6 production in an ERK1/2-dependent manner. Of note, as C5aR2 itself can induce ERK1/2 activation, the effects of P32/P59 on C5aR2-mediated MAPK activity (versus that driven by C5aR1 and/or TLR4 stimulation) remain to be dissected. In neutrophils (right half), P32/P59 binding to C5aR2 diminishes C5aR1-driven cell mobilization *in vivo*. Whether this occurs also through blockage of ERK1/2 activation remains to be tested (question mark).

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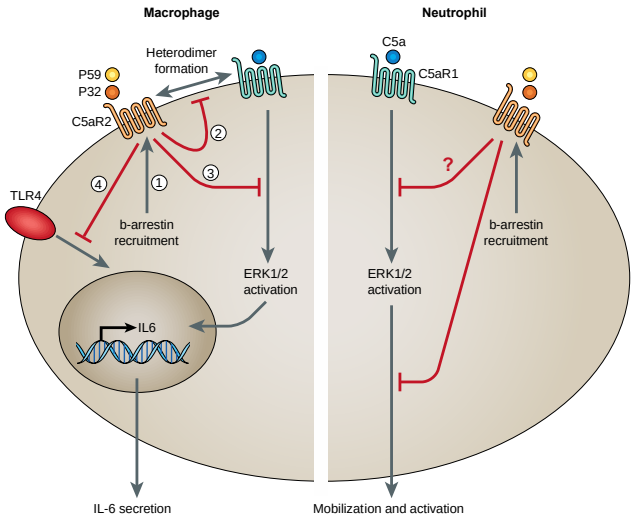
Figure 1.


Macrophage
Neutrophil
Heterodimer formation
P59
P32
C5aR2
C5a
C5aR1
TLR4
b-arrestin recruitment
ERK1/2 activation
IL6
IL-6 secretion
Mobilization and activation
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